Zebrafish Models of Anxiety-Like Behaviors

Adam D. Collier, Allan V. Kalueff, and David J. Echevarria

Abstract Anxiety disorders are widespread psychiatric illnesses affecting approximately 7–10 % of the global population. Zebrafish are a particularly useful animal model for studying anxiety-related phenotypes. They are increasingly utilized for studying neurobiological, physiological and genetic mechanisms of anxiety, as well as for screening various anxiolytic drugs. Summarized here, accumulating evidence supports the utility of zebrafish neurobehavioral phenotyping in studying anxiety and stress neurobiology. For example, zebrafish are highly sensitive to various anxiety-evoking environmental stressors, including novelty, predator exposure, alarm pheromone, anxiogenic drugs, and drug withdrawal. Zebrafish also show high sensitivity to anxiolytic manipulations. Zebrafish anxiety-related neuroendocrine responses are also robust, sensitive, and correlate strongly (and bi-directionally) with behavioral endpoints. Finally, zebrafish are also amenable to genetic manipulations, and differences in baseline and experimentally-evoked anxiety levels can be observed in different strains of zebrafish. Collectively, this supports the validity and efficiency of both larval and adult zebrafish model for studying acute and chronic anxiety-like states.

Keywords Zebrafish • Anxiety • Stress • Endocrine response • Cortisol • Novelty • Novel tank • Open field • Light dark • Predator stress • Alarm pheromone • Strain differences • Behavioral phenotyping

A.D. Collier • D.J. Echevarria
Department of Psychology, University of Southern Mississippi, Hattiesburg, MS, USA

The International Zebrafish Neuroscience Research Consortium (ZNRC), Slidell, LA, USA

A.V. Kalueff (✉)
The International Zebrafish Neurosceince Research Consortium (ZNRC) and ZENEREI Research Center, Slidell, LA, USA

Research Institute of Marine Drugs and Nutrition, College of Food Science and Technology, Guangdong Ocean University, Zhanjiang, Guangdong, China

Institute of Translational Biomedicine, St. Petersburg State University, St. Petersburg, Russia

Institutes of Chemical Technology and Natural Sciences, Ural Federal University, Ekaterinburg, Russia
e-mail: avkalueff@gmail.com

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1 Introduction

Anxiety disorders are common psychiatric illnesses that involve multifaceted interactions between behavior, neural circuitry, physiology, genetics and experience [1]. Anxiety disorders are particularly widespread, affecting approximately 7–10 % of the general population [2]. Clinical manifestations of anxiety-related disorders are summarized in Table 1 [3].

An important strategy to elucidate neural underpinnings and develop novel treatments is to study animal models which have highly conserved neural circuitry related to anxiety [4]. A variety of behavioral paradigms, pharmacological screens, and genetic manipulations in animal models especially rodents, have long been employed to study anxiety disorder pathogenesis [5–7]. However, experimental rodent models are often low-throughput, costly and time-consuming [8]. The zebrafish (*Danio rerio*) has emerged as a new advantageous in-vivo preclinical model organism used in biomedical and translational neuroscience research to study the behavioral and molecular mechanisms underlying brain disorders, including anxiety. As will be shown in this and other chapters of this book, zebrafish display complex and well-defined behavioral phenotypes [9] (also see chapter "Illustrated Zebrafish Neurobehavioral Glossary" in this Book), and are amenable to high-throughput screening due to low-cost and small size [10, 11]. Video tracking technologies are also readily available to be coupled with zebrafish behavioral assays, providing data-rich endpoints (e.g., velocity, distance traveled) and 'big data'-level analyses (e.g., three-dimensional spatial and spatiotemporal swim path reconstructions, behavioral barcoding approaches) that are impossible to generate manually [12–14]. Zebrafish also possess all major neurotransmitter systems, transporters, receptors and hormones [15–17], and have a fully sequenced genome with ~70–75 % of human genes having at least one zebrafish orthologue [18].

Zebrafish are rapidly becoming a promising model organism for anxiety and stress research, especially due to a robust and easily quantifiable cortisol stress response [15, 19], clear-cut drug-evoked phenotypes with high predictive validity [20, 21], and fish sensitivity to a wide range of experimental stressors. For example, like with rodents, stressors that can trigger zebrafish anxiety-like behaviors include novelty exposure [21], social isolation and confinement [8], predator exposure [22] and alarm substance [23]. Furthermore, a number of genetic strains that show behavioral differences are now available [24], with multiple cutting-edge genome editing tools that can be applied to zebrafish [25–27]. Here, we introduce several behavioral paradigms and outline aspects of zebrafish phenotyping related to anxiety-like states. Focusing mainly on adult zebrafish models with established neural and physiological systems, this chapter also briefly mentions conceptually similar approaches to model anxiety-like states in larval zebrafish.

Table 1 Summary of clinical symptoms of anxiety-related disorders and diagnostic criteria from DSM, Diagnostic and Statistical Manual of Mental Disorders, 5th edition [3]

Anxiety disorders	Potentially relevant zebrafish phenotypes
Generalized anxiety disorder (GAD)	
Excessive anxiety/worry about events and activities	Anxiety-like behaviors
Difficulty controlling worry	–
Restlessness	Hyper-arrousal, erratic movements
Fatigue	Fatigue
Difficulty concentrating	Poor performance in cognitive tests
Irritability	Increased social aggression
Muscle tension	–
Sleep disturbance	Sleep deficits
The anxiety, worry, or physical symptoms causes clinically significant distress and impairs general functioning (e.g., social, occupational)	Anxiety behaviors in various contexts, aversive conditioning
Specific phobia	
Marked and persistent fear or anxiety about a specific object or situation	Anxiety behaviors in various contexts
Avoidance of phobic object or situation	Neophobia
Fear or anxiety out of proportion to actual danger posed by specific object or situation	Anxiety behaviors in various contexts, aversive conditioning
Fear and anxiety of specific object or situation causes clinically significant distress and impairs general functioning (e.g., social, occupational)	Anxiety behaviors in various contexts, aversive conditioning
Social anxiety disorder	
Marked and persistent fear or anxiety about social situations	Social deficits
Fear of showing anxiety symptoms that will be negatively evaluated	Increased anxiety in social interactions or contexts/tests
Avoidance of social situations or endured with intense fear and anxiety	Anxiety behaviors in various contexts, aversive conditioning, social avoidance
Fear and anxiety out of proportion to actual danger posed by social situation	Anxiety behaviors in various contexts, aversive conditioning, social avoidance
Fear and anxiety of social situation causes clinically significant distress and impairs general functioning (e.g., social, occupational)	Anxiety behaviors in various contexts, aversive conditioning, social avoidance
Panic disorder	
Recurrent unexpected panic attacks	Increased anxiety
Accelerated heart rate	Accelerated heart rate

(continued)

Table 1 (continued)

Anxiety disorders	Potentially relevant zebrafish phenotypes
Sweating	–
Trembling or shaking	Trembling or shaking
Shortness of breath	Shortness of breath
Feelings of choking	–
Chest pain or discomfort	–
Nausea or abdominal distress	Nausea or abdominal distress
Feeling dizzy/faint	–
Chills or heat sensations	–
Paresthesias	–
Derealization/Depersonalization	–
Fear of losing control	–
Fear of dying	–
Agoraphobia	
Marked and persistent fear and anxiety about agoraphobic situations (e.g., public transportation, being in open spaces, being in enclosed places, being in a crowd, being outside of the home alone)	Increased thigmotaxis and aversive conditioning
Avoidance of agoraphobic situations	–
Fear and anxiety of agoraphobic situation is out of proportion to actual danger	–
Agoraphobic situation causes clinically significant distress and impairs general functioning (e.g., social, occupational)	–
Substance/medication-induced anxiety disorder	
Panic attacks or anxiety	Increased anxiety behaviors
Evidence that panic attacks or anxiety developed during or soon after substance intoxication or withdrawal	Pharmacogenic or withdrawal-evoked anxiety
Evidence that the substance is capable of producing panic attacks or anxiety	Pharmacogenic or withdrawal-evoked anxiety
The disturbance is not better explained by another anxiety disorder	–
The disturbance causes clinically significant distress and impairs general functioning (e.g., social, occupational)	

2 Novelty-Based Behavioral Paradigms: Open Field, Light Dark, and Novel Tank Tests

Traditionally, animal models of anxiety are often based on behavioral responses to novel environments [28, 29]. In many taxa, exposure to a novel (and, therefore, potentially dangerous) environment often triggers the expression of avoidance-related behaviors in animals that likely serve evolutionarily conserved 'anti-predatory' functions [30, 31]. Novelty exploration is believed to underlie behavioral

organization in a new environment and reflect the emotional state of animals [32–34]. Typical 'spatial' behaviors include total distance traveled, average velocity, and spatial distribution of exploratory activity. Initial exploratory behaviors tend to attenuate over the testing session as animals habituate to novel environments, a form of non-associative learning and an important cognitive phenotype, the impairment of which may be associated with increased anxiety [35, 36]. Like in rodents, zebrafish novelty-based paradigms and associated phenotypes are highly sensitive to exposure to acute and chronic stressors and pharmacolological manipulations (Tables 2, 3, 4, and 5), and can therefore can be used to screen drug effects [51, 58]. Accordingly, a number of novelty-based paradigms traditionally developed and used for rodents, have been applied to zebrafish neurophenotyping.

One of the most popular animal behavioral paradigms is the open-field test, which since its invention in 1932 is most commonly used in rodents to evaluate their novelty-evoked anxiety-like behaviors [29, 59–61]. Recently, this paradigm has been adapted to neurobehavioral phenotyping of both larval [62] and adult zebrafish [34], and its typical exploratory-based behavioral endpoints include the time in/entries to the center, time in/entries to the periphery (i.e., thigmotaxis), distance traveled and average velocity (Table 2). The center and periphery of the open field apparatus may be defined differently across laboratories. For example, one group could visually divide the open field into 16 equally sized squares and define the center as the middle 4 squares and the periphery as the remaining outer squares [36]. In other studies, center can be defined arbitrarily as area within 5 cm from the walls of the apparatus [37]. There are no specific standards regarding how to best define

Table 2 Adult zebrafish anxiety-related behavioral phenotypes: the open field test

Phenotype	Treatment + effect	↑ Value indicates	References
Entries to center	Acute LSD ↓	↑ entries to center indicates ↓ anxiety	[36, 37]
Time in center	Acute LSD Ø	↑ time in center indicates ↓ anxiety	[37]
	α-fluoro-methylhistidine ↑		
Entries to periphery	Acute LSD Ø	↑ entries to periphery indicates ↑ anxiety	[37, 38]
Time in periphery	Acute LSD Ø	↑ time in periphery indicates ↑ anxiety	[37]
Distance traveled in periphery	Cocaine withdrawal ↑	↑ distance traveled in periphery indicates ↑ anxiety	[39]
	FG-7142 ↑		
Distance traveled (total)	Acute LSD Ø	↑ total distance traveled indicates hyperactivity	[13, 37, 40]
	Acute ibogaine Ø		
	FG-7142 ↑		
Average velocity	Acute LSD Ø	↑ average velocity indicates motor aspects of zebrafish swimming	[13, 37]
	Acute ibogaine Ø		

↑ increased/activated, ↓ reduced/inhibited/impaired, Ø no effect, *LSD* lysergic acid diethylamide, *FG-7142* a benzodiazepine antagonist

Table 3 Adult zebrafish anxiety-related behavioral phenotypes: the light dark test

Phenotype	Treatment + effect	↑ Value indicates	References
Latency to dark side	CUS ↓	↑ latency to enter the dark side indicates ↓ anxiety	[36, 37, 41, 42]
	Acute LSD Ø		
	Acute ibogaine ↑		
	Acute ketamine ↑		
Time in dark side	CUS ↑	↑ time in the dark side indicates ↑ anxiety	[36, 37, 41–44]
	Acute LSD ↓		
	Restraint stress ↑		
	Acute ibogaine ↓		
	Acute caffeine ↑		
	Acute ZM241385 Ø		
	Acute DPCPX ↑		
	Acute fluoxetine Ø		
	Chronic fluoxetine ↓		
	Acute CDP ↓		
	Acute clonazepam ↓		
	Acute diazepam ↓		
	Acute buspirone ↓		
	Acute moclobemide Ø		
	Acute ethanol ↓		
	Acute ketamine ↓		
Entries to dark side	Acute LSD Ø	↑ entries to the dark side indicates ↑ anxiety	[13, 36, 37]
	Restraint stress Ø		
	Acute ibogaine ↓		
Average dark side entry duration	Acute LSD Ø	↑ dark side entry duration indicates ↓ anxiety	[13, 37, 42]
	Acute ibogaine Ø		
	Acute ketamine Ø		
Midline crossings	Chronic fluoxetine Ø	↑ midline crossings indicates ↑ swimming activity	[42, 44]
	Acute CDP Ø		
	Acute clonazepam ↓		
	Acute diazepam Ø		
	Acute buspirone ↓		
	Acute moclobemide Ø		
	Acute ethanol ↑		
	Acute caffeine Ø		
	Acute ketamine ↑		

↑ increased/activated, ↓ reduced/inhibited/impaired, Ø no effect, *CUS* chronic unpredictable stress, *LSD* lysergic acid diethylamide, *CDP* chlordiazepoxide, *ZM241385* an adenosine A_2 antagonist, *DPCPX* an adenosine A_1 antagonist

Table 4 Adult zebrafish anxiety-related behavioral phenotypes: the novel tank test

Phenotype	Treatment + effect	↑ Value indicates	References
Latency to upper half	Chronic fluoxetine ↓	↑anxiety	[24, 41, 45–50]
	Acute alarm pheromone ↑		
	Acute MDMA ↓		
	Acute caffeine Ø		
	Acute ethanol ↓		
	Chronic ethanol Ø		
	Leopard strain ↑		
	Wild-derived Indian strain ↑		
	CUS ↑		
	Acute nicotine ↓		
	Chronic nicotine ↑		
	Acute PCP ↓		
	Acute ketamine ↓		
	Acute ibogaine ↓		
	Acute noribogaine ↓[a]		
	Chronic CDP Ø		
	CDP withdrawal ↑		
	Vmat2 knockdown ↑		
Entries to upper half	Chronic fluoxetine ↑	↓ anxiety	[24, 41, 45–50]
	Acute alarm pheromone ↓		
	Acute MDMA ↓		
	Acute caffeine ↓		
	Acute ethanol ↑		
	Chronic ethanol ↑		
	CUS ↓		
	Wild-derived Indian strain ↓		
	Acute nicotine Ø		
	Chronic nicotine ↓		
	Acute PCP Ø		
	Acute ketamine ↑		
	Acute ibogaine Ø (↑ in first 2 min)		
	Acute MK-801 Ø		
	Chronic CDP Ø		
	CDP withdrawal ↓		
	Vmat2 knockdown ↓		

(continued)

Table 4 (continued)

Phenotype	Treatment + effect	↑ Value indicates	References
Time in upper half	Chronic fluoxetine ↑	↓ anxiety	[24, 41, 45–52]
	Acute alarm pheromone ↓		
	Acute MDMA ↑		
	Acute caffeine ↓		
	Acute ethanol ↑		
	Chronic ethanol ↑		
	Leopard strain ↓		
	Wild-derived Indian strain ↓		
	CUS ↓		
	Acute nicotine ↑		
	Chronic nicotine ↓		
	Acute PCP Ø		
	Acute ketamine ↓		
	Acute ibogaine Ø		
	Acute noribogaine ↑[a]		
	MK-801 ↑		
	Acute buspirone ↑		
	Acute CDP Ø		
	Chronic CDP Ø		
	CDP withdrawal ↓		
	Acute diazepam ↑		
	Vmat2 knockdown ↓		
Erratic movements	Chronic fluoxetine ↓	↑ anxiety	[24, 47]
	Acute alarm pheromone ↑		
	Acute MDMA ↓		
	Acute caffeine Ø		
	Acute ethanol Ø		
	Acute ketamine Ø		
	Acute ibogaine ↑		
	Acute MK-801 Ø		
Freezing bouts	Acute alarm pheromone ↑	↑ anxiety	[24, 45–50, 53]
	Acute MDMA Ø		
	Chronic nicotine Ø		
	Acute PCP ↑		
	Wild-derived Indian strain ↑		
	Acute ketamine Ø		
	Acute ibogaine Ø or ↑		
	Acute MK-801 Ø		
	Chronic CDP Ø		
	CDP withdrawal Ø		
	Vmat2 knockdown Ø		

(continued)

Table 4 (continued)

Phenotype	Treatment + effect	↑ Value indicates	References
Distance traveled (total)	Chronic fluoxetine Ø	↑ hyperactivity or increased exploration	[24, 49]
	Chronic ethanol Ø		
	Acute PCP Ø		
	Acute ketamine Ø		
	Acute ibogaine Ø or ↑		
	Acute MK-801 ↑		
Average velocity	Chronic fluoxetine Ø	Various motor aspects of zebrafish swimming	[24, 49, 51, 52]
	Chronic ethanol Ø		
	Acute PCP Ø		
	Acute ketamine Ø		
	Acute ibogaine Ø		
	MK-801 ↑		
	Acute nicotine ↑		
	Acute buspirone Ø		
	Acute CDP ↓		
	Acute diazepam Ø		
	Vmat2 knockdown ↑		

↑ increased/activated, ↓ reduced/inhibited/impaired, Ø no effect, *MDMA* 3,4-methylenedioxymethamphetamine, *CUS* chronic unpredictable stress, *PCP* phencyclidine, *CDP* chlordiazepoxide, *LSD* lysergic acid diethylamide, *MK-801* dizoclipine, *Vmat2* vesicular monoamine transporter 2
[a]Unpublished data (Maillet, Kalueff, 2015, DemeRx LLC)

Table 5 Larval zebrafish anxiety-related behavioral phenotypes

Phenotype	Treatments + effects	↑ Value indicates	References
Open field test			
Time in periphery	Acute ethanol ↑	↑ time in periphery indicates ↑ anxiety	[54]
	Acute diazepam ↓		
	Acute caffeine ↑		
Distance traveled in periphery	Acute diazepam ↓	↑ distance moved in periphery indicates ↑ anxiety	[55]
	Acute caffeine ↑		
Distance traveled (total)	Acute diazepam Ø	↑ total distance traveled indicates hyperactivity	[56]
	Acute caffeine Ø		
Light dark test			
Latency to dark side	Diazepam ↓	↑ latency to enter the dark side indicates ↑ anxiety	[57]
	Buspirone ↓		
	Ethanol ↓		
	Caffeine ↑		
Time in dark side	Diazepam ↑	↑ time in the dark side indicates ↓ anxiety	[57]
	Buspirone ↑		
	Ethanol ↑		
	Caffeine ↓		
Entries to dark side	Diazepam Ø	↑ entries to the dark side indicates ↓ anxiety	[57]
	Buspirone ↑		
	Ethanol ↑		
	Caffeine ↓		

↑ increased/activated, ↓ reduced/inhibited/impaired, Ø no effect

the center in the open field test. Thus, well-defined zones in this test must be consistent and standardized within the laboratory, to ensure valid behavioral phenotypic data. Also importantly, both rodents and zebrafish initially exhibit thigmotaxic anxiety-like behaviors during open field testing, which decrease over time, indicative of intra-session habituation to novelty [63, 64].

Furthermore, although the open field studies are similar in that they each evaluate exploratory behavior when placed into a novel and open environment, differences often exist across laboratories in testing duration, pretest housing conditions, and the size, shape, color and texture of the apparatus (Fig. 1). Zebrafish increase locomotor behavior in a larger open field arena compared to a smaller arena, but, interestingly, the overall temporal activity patterns for their exploratory behaviors remain stable across different arena sizes [34]. Similarly, rodents display differential locomotor behavior depending on the size of the arena, and exhibit a temporal stability in activity

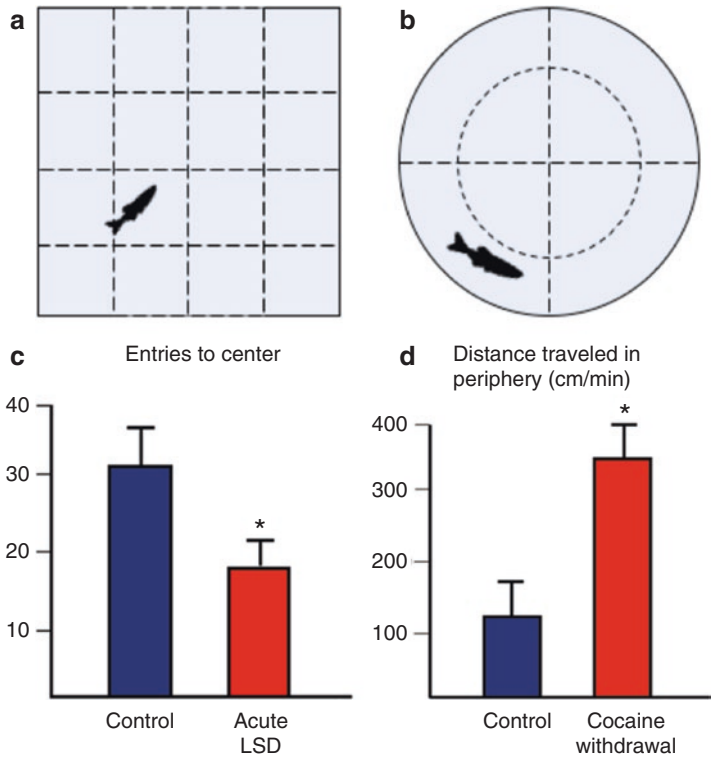

Fig. 1 The zebrafish open-field test (*top view*). This paradigm primarily evaluates horizontal exploratory based behavioral endpoints, such as entries/time spent in the center zones, entries/time spent in the outer zones (i.e., thigmotaxis), total distance traveled, and average velocity. (**a**) A square open field apparatus divided into zones [36]. (**b**) A circular open field apparatus divided into zones [13]. (**c**) Acute lysergic acid diethylamide (LSD) exposure decreased the number of entries to the center of the open field [37]. (**D**) 72-h withdrawal from cocaine (5 intermittent days 1.5 μM) increased distance traveled in the periphery [39]

throughout testing, suggesting that novelty exploration behavior in the open field is well conserved in zebrafish [65, 66]. As already mentioned, like rodent models, zebrafish readily habituate to the open field over time as indicated by a reduction in distance traveled and average velocity by the end of the testing session [36].

Another common phenotype observed during rodent open field testing is the establishment of a homebase, a preferred reference point location commonly seen in rodents [67, 68], which was recently reported in zebrafish [69]. Zebrafish homebase behavior can be measured by dividing the open field arena into quadrants and quantifying average time spent, frequency of visits and distance traveled in each quadrant [69]. This behavior can be sensitive to pharmacological manipulation, since for example, a hallucinogenic drug ibogaine reduces the time spent investigating the entire open field arena before establishing a preferred homebase behavior compared to control fish [13]. Other exploratory behaviors in the open field are sensitive to pharmacological treatment as well, and are summarized in Table 2. Furthermore, open field phenotypes are also sensitive to experimental stressors. For example, an acute stressor such as a 15-min net restraint increases thigmotaxis and average velocity in zebrafish [36].

The light dark test is another paradigm traditionally tested in rodents, and currently extensively applied to zebrafish phenotyping. The light dark test apparatus is typically an aquarium that consists of a light half and a dark half [36, 37, 43]. The test can also take other forms, such as the light dark plus maze with a grey center starting area with two light and two dark arms (Fig. 2) [71]. Rodents are innately aversive to brightly lit environments and exhibit scototaxis (i.e., dark environment preference); a decrease in scototactic behavior indicates anxiolysis [72, 73]. Similarly, adult zebrafish, as well as other fish species (e.g., goldfish, guppies, minnows and tilapia), generally display a robust preference for the dark area of the tank [13, 37, 43, 44]. However, there are early reports of a preference for the white area of the tank in zebrafish [36, 74]. These reported inconsistencies are likely attributable to different housing conditions, lighting, fish sex, age, social status and/or strains, and can be interpreted carefully, keeping in mind a marked and common preference for dark in normal adult zebrafish (and 'reversed' light preference in larval fish [9]). For example, 2 months of rearing in an enriched environment increased time spent in the light environment compared to fish raised in an impoverished environment [75]. Differences in lighting intensity can also alter zebrafish behavior in the light dark test; zebrafish increased scototaxis and spent more time freezing at 500 vs. 250 lux [43]. Further experimentation is necessary to elucidate the factors responsible for differences in baseline scototaxic behavior. The light dark test has been commonly employed with rodent models to evaluate stressor and drug effects on anxiety-related phenotypes [72]. Zebrafish scototaxis is also bidirectionally sensitive to screen such effects (Table 3) [20]. For instance, chronic fluoxetine (an antidepressant with anxiolytic action) and acute benzodiazepine anxiolytics (i.e., chlordiazepoxide, clonazepam and diazepam) all decrease scototaxis [44], while acute caffeine, acute restraint stress and chronic unpredictable stress (CUS) increase scototaxis [36, 41, 44].

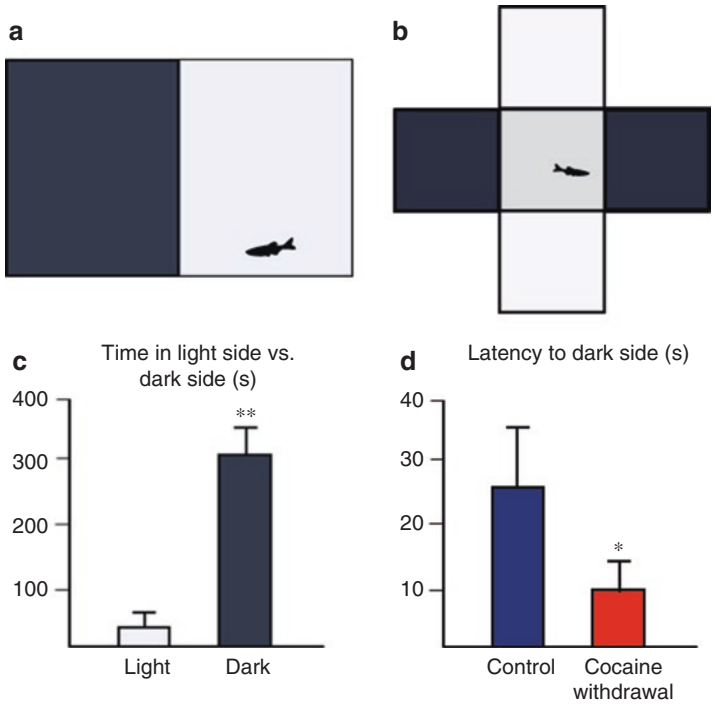

Fig. 2 The zebrafish light dark test. This paradigm primarily evaluates changes in horizontal exploration of light and dark environments, such as the duration of time, number of entries and latency to enter each half. (**a**) A typical light dark test apparatus (*side view*), an aquarium tank with a light colored half and a dark colored half [70]. (**b**) An alternative light dark test apparatus (*top view*) represents a plus maze with a grey center and two light arms and two dark arms [71]. (**c**) Zebrafish commonly display a baseline preference for the darker environment over the light environment (i.e., scototaxis) [70]. (**d**) CUS (chronic unpredictable stress) for 15 days decreased latency to enter the dark side [41]

The novel tank test is a popular novelty-based paradigm that is unique to zebrafish and other aquatic species, and is often used for their behavioral phenotyping and testing drug effects. This test is conceptually similar to the open field test used for rodents, but rather than measuring only horizontal exploration, the novel tank task primarily measures vertical exploration [70]. The novel tank apparatus typically consists of a narrow tank divided horizontally into a top and bottom zone, but may also consist of a three-zone tank (i.e., top, middle and bottom zones; Fig. 3). Upon exposure to a novel tank apparatus, zebrafish initially exhibit a robust anxiety-like response by diving to the bottom of the tank (i.e., geotaxis), also reducing exploration, increasing freezing and erratic movements [21]. Additionally, this paradigm induces stress-related physiological responses, such as elevated cortisol levels, increased breathing and increased heart beat frequency [45]. Habituation to the novel tank occurs over time, as indicated by a decrease in the aforementioned anxiety phenotypes [35]. It is important to note that pre-test housing conditions may

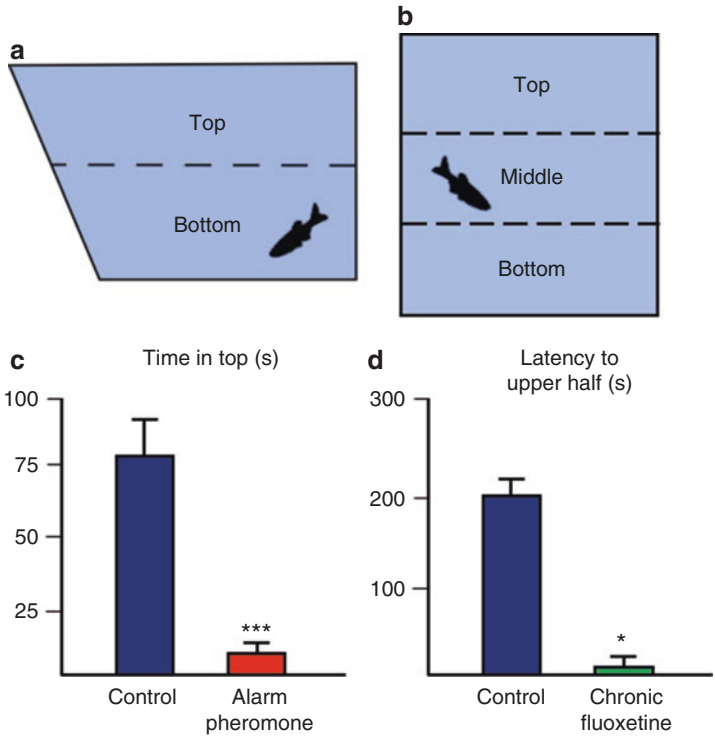

Fig. 3 The zebrafish novel tank test (*side views*). This paradigm primarily evaluates changes in vertical exploration, such as time spent in top and bottom zones, number of entries, latency to enter the top, total distance traveled, and average velocity. Zebrafish initially dive to the bottom of the tank (i.e., geotaxis) and explore upper regions of the tank as habituation occurs (**a**) A typical novel tank test apparatus, consisting of a trapezoidal tank divided into top and bottom zones [70]. (**b**) An alternative novel tank apparatus, divided into top, middle, and bottom zones [71]. Generally, both modifications of this model will be sensitive to zebrafish anxiety-like behaviors, albeit the central zone in model B would mostly reflect a transition aspect between two other zones (top/bottom, the difference between which would be both more relevant behaviorally and most robust phenotypically). (**c**) Anxiogenic effect of acute alarm pheromone on zebrafish behavior in the novel tank test [76]. (**d**) Anxiolytic effect of chronic fluoxetine (100 g/L 2 weeks) in the novel tank test [24]

affect zebrafish behavior in this paradigm. For instance, zebrafish housed in a narrow tank similar to the novel tank apparatus may not display a diving response or changes in swim velocity, but fish housed in a wider tank did, an effect that was likely due to habituation or acclimation to novelty [51].

The novel tank test is an excellent assay for screening anxiotropic (anxiolytic and anxiogenic) agents, as zebrafish anxiety-like behaviors are highly and bidirectionally sensitive to such manipulations (Table 4). For example, chronic fluoxetine reduces, and acute caffeine increases geotaxis [21], similar to these drugs' effects in rodents. The novel tank test can also be used to evaluate anxiety phenotypes evoked experimentally by drug withdrawal (Table 4). Specifically, repeated morphine withdrawal in zebrafish produces a robust anxiogenic profile in the novel tank test in

zebrafish [77]. Similar anxiogenic effects of withdrawal are observed in rodent models as well, lending further credence to zebrafish models of withdrawal-evoked anxiety [78]. Additionally, experimental stressors and strain differences produce altered zebrafish anxiety and locomotor phenotypes in this paradigm (Table 4).

Finally, larval zebrafish also show similar behavioral responses to anxiolytic and anxiogenic stimuli in novelty-based paradigms (Table 5), albeit their natural preference for light (scotophobia) 'inverts' the interpretation of the light dark box data, and is gradually replaced with normal photophobia/scototaxis as adults [9]. Acute diazepam, ethanol and buspirone produce anxiolytic responses in the larval light dark test, as indicted by, for example, increased time in the dark side [57]. Conversely, acute caffeine produces an anxiogenic response in the light dark test [57]. Larval zebrafish display characteristic thigmotaxis and avoidance of the center region in the open field test [62]. The larval apparatus may vary in shape, size and color, but typically consists of a 12 or 24-well plate with each well visually divided into an inner and outer zone [55]. Thigmotaxis is enhanced by caffeine and potentiated by diazepam, thus validating the sensitivity of larval zebrafish to study anxiety-like behaviors in the open field.

In summary, each of these behavioral tests do not involve training, are short in experimental duration (usually 5–10 min), and are relatively simple to employ. This, coupled with the advantageous characteristics of adult and larval zebrafish model, provides an ideal scenario for many experimental applications, including high-throughput phenotyping, gene and drug screening relevant to anxiety.

3 Physiological (Endocrine) Response: Cortisol

Robust and quantifiable physiological phenotypes contribute markedly to the utility of zebrafish models for stress and anxiety research. The zebrafish hypothalamus-pituitary-interrenal (HPI) axis is homologous to the human hypothalamus-pituitary-adrenal (HPA) axis, with cortisol being the primary stress hormone in both species (Fig. 4). The evolutionarily conserved stress response between zebrafish and humans establish this aquatic species as a valid model to study cortisol-mediated stress responses [15, 24]. Cortisol can be sampled using different methods, using adult whole-body samples [79], tail vein blood and trunk samples [80], testing water [81], and whole-body larval zebrafish [82]. A temporal-based analysis of whole-body cortisol levels following a net stressor (i.e., acute net handling and air exposure) found increased cortisol at 3 min post-stressor, a linear increase and peak levels at 15 min post-stressor, and return to near control levels 60 min post-stressor [83] (Fig. 5). The analysis of neuroendocrine (i.e., cortisol) responses in zebrafish is a valuable tool complementing behavioral studies. Zebrafish modulate cortisol levels in response to various drug treatments and experimental stressors (Table 6), which often strongly correlate with behavioral responses [21]. For instance, zebrafish treated with chronic fluoxetine decreased whole-body cortisol levels (Fig. 5) and reduced geotaxis in the novel tank test [21]. Conversely, anxiogenic manipulations like morphine withdrawal increased both whole-body cortisol and geotaxis in the novel tank test [77].

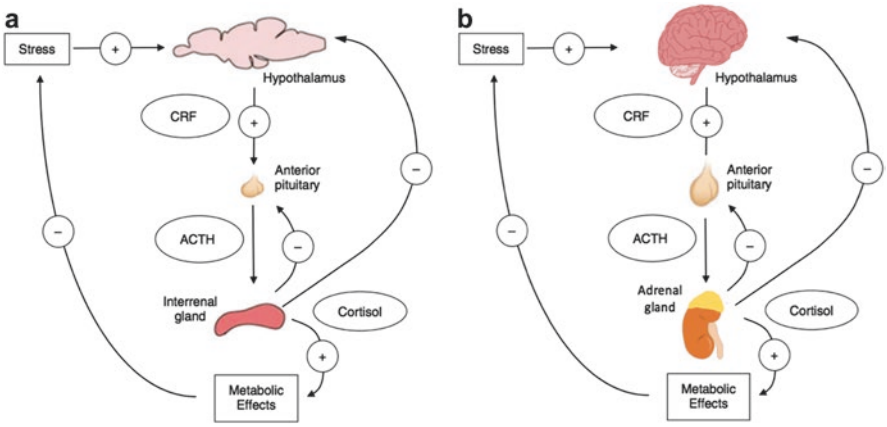

Fig. 4 A striking overall physiological similarity of the endocrine stress axes in zebrafish and humans. "+": activation. "–": inhibition. *CRF* corticotropin-releasing factor, *ACTH* adrenocorticotropic hormone. (**a**) Zebrafish hypothalamus-pituitary-interrenal (HPI) axis. (**b**) Human hypothalamus-pituitary-adrenal (HPA) axis [76]

4 Experimental Stressors: Chronic Unpredictable Stress, Beaker Stress, Predator and Alarm Pheromone Exposure

Zebrafish behavioral and physiological phenotypes are highly sensitive to acute or chronic exposure to a wide range of husbandry, environmental, chemical, mechanical and social stressors (e.g., changes in temperature, pH and lighting, crowding, isolation, restraint, decreasing water level, chasing with net, air exposure, dominant and submissive pairings, predator exposure, and alarm pheromone exposure) [19, 41, 88]. Chronic unpredictable stress (CUS), consisting of a battery of stressors administered over a length of days (see Table 6 for details), increased whole-body cortisol levels as well as anxiety-like behaviors in the novel tank test and light dark test [41]. CUS also down-regulated phosphorylated cAMP response element-binding protein (pCREB), up-regulated corticotropin-releasing factor (CRF) as well as calcineurin mRNA in the zebrafish brain, which are molecular markers that have been observed in human patients with major depressive disorder and rodent models of mood disorders [89–91]. However, brain derived neurotrophic factor (BDNF) is up-regulated in zebrafish following CUS [41], whereas it is commonly down-regulated in rodent models [92, 93]. Notably, BDNF levels are differentially expressed in the rat amgydala and hippocampus, and therefore, it may be useful for future studies to evaluate zebrafish gene expression profiles in a brain region-specific manner [94]. An upregulation of several other molecular markers related to the HPI axis, such as whole brain glucocorticoid receptor (GR), mineralcorticoid receptor (MR), proopiomelanocortin (POMC) hypocretin/orexin, BDNF, as well as c-fos mRNA, has been reported in zebrafish [19]. The immediate early gene *c-fos* acts a reliable biomarker of cellular (e.g., neuronal) activation in various species, including humans [95], rodents [96] and zebrafish [97, 98].

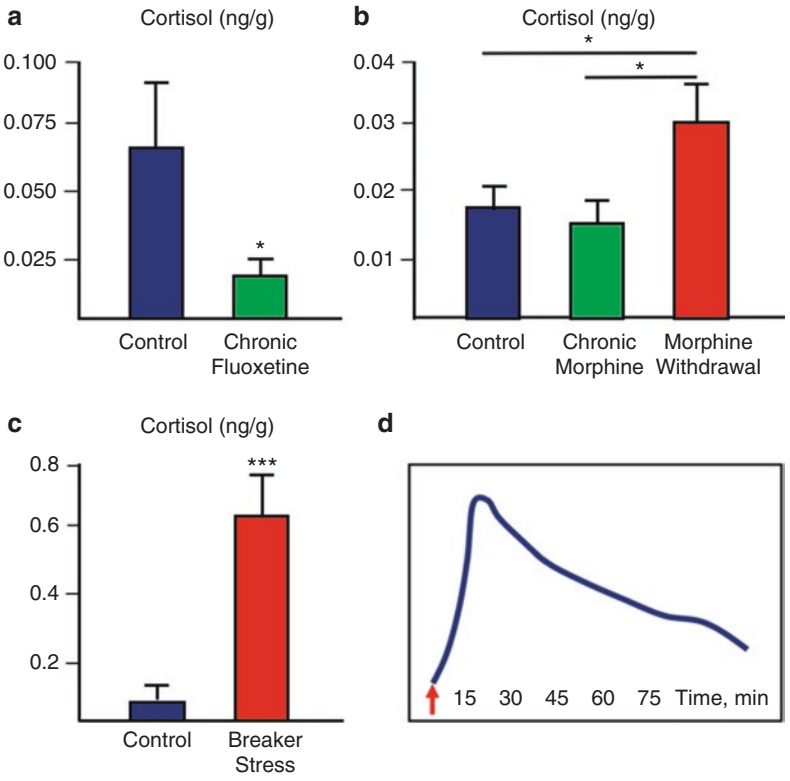

Fig. 5 A typical zebrafish cortisol responses (whole-body cortisol, ng/g fish). (**a**) Exposure to chronic fluoxetine (2 week 100 μg/L). (**b**) Exposure to chronic morphine (2 week 1.5 mg/L) and 24-h morphine withdrawal. (**c**) Exposure to beaker stress paradigm (15 min at 100 mL water in 250 mL beaker). Data are presented as mean ± SEM, *$p<0.05$, **$p<0.01$, *** $p<0.001$. *U*-test (Modified from [8, 21]). (**d**) A typical time course of zebrafish stress-evoked cortisol response, peaking at 15 min after a stress stimulus (*arrow*) and gradually decreasing over a 1–1.5-h time [83]. Note that this response strikingly resembles the dynamic of human cortisol response to acute stressor

Acute environmental stressors also modulate zebrafish anxiety phenotypes. For example, a recently developed beaker stress model, consisting of confinement for 15 min in 100 mL of water in a 250 mL glass beaker, robustly increases anxiety-like behaviors in the novel tank test and light dark test (own unpublished findings) and whole-body cortisol levels (Fig. 6, Table 6), also see [8]. The robustness of this model is likely due to the combination of confinement in a small environment, a shallow <10 cm water level (stressful for zebrafish), and social isolation from conspecifics. Alarm pheromone exposure also produces behavioral alterations in zebrafish, released by their injured skin cells and detected by the fish olfactory system [23]. Alarm pheromone can be easily extracted from the epidermal cells of euthanized zebrafish and administered to tank water [23]. Acute alarm pheromone

Table 6 Summary of zebrafish cortisol responses to various stimuli

Treatment	Details	Cortisol effect	References
Acute LSD	20-min 250 μg/L	↑ vs. control	[37]
Acute PCP	20-min 3 mg/L	↑ vs. control	[84]
Acute mescaline	20-min 20 mg/L	Ø vs. control	[84]
Acute ibogaine	20-min 10 + 20 mg/L	Ø vs. control	[13]
Acute ketamine	20-min 20 mg/L + 40 mg/L	↓ vs. control	
Chronic nicotine	4 days (2 days 1 mg/L + 2 days 2 mg/L)	Ø vs. control	[48]
Chronic fluoxetine	2 week 100 μg/L	↓ vs. control	[21]
Chronic morphine	2 week 1.5 mg/L	Ø vs. control	[21]
Morphine withdrawal	24-h withdrawal from chronic treatment	↑ vs. control	[21]
		↑ vs. chronic treatment	
Chronic ethanol	1 week 0.3 % v/v	Ø vs. control	[21]
Ethanol withdrawal	24-h withdrawal from chronic treatment	Ø vs. control	[21]
		↑ vs. chronic treatment	
Chronic diazepam	2 week 72 mg/L	Ø vs. control	[77]
Diazepam withdrawal	72-h withdrawal from chronic treatment	Ø vs. control	[77]
		Ø vs. chronic treatment	
Chronic caffeine	1 week 50 mg/L	Ø vs. control	[77]
Caffeine withdrawal	12-h withdrawal from chronic treatment	Ø vs. control	[77]
		Ø vs. chronic treatment	
Chronic CDP	4-month 100 mg/L	Ø vs. control	[50]
CDP withdrawal	7-day CDP withdrawal from chronic treatment	↑ vs. control	[50]
		↑ vs. chronic treatment	
Dyadic social stress	Dominant and submissive fish kept in pairs for 5 days	↑ in dominant fish vs. control	[80]
		↑ in submissive fish vs. control	
Predator exposure (direct contact)	5 min of *Parachromis managuensis* exposure	↑ vs. control	[85]
Predator exposure (visual contact)	60 min of *Parachromis managuensis* exposure	↑ vs. control	[85]
Beaker stressor	15 min in 100 mL within a 250 mL beaker	↑ vs. control	[8]

(continued)

Table 6 (continued)

Treatment	Details	Cortisol effect	References
Acute net handling	3 min net suspension in air + 3 min in tank	↑ vs. control	[83]
	$\frac{+3\,\text{min suspension in air}}{30\,\text{s net suspension in air}}$	↑ vs. control	[86]
Acute crowding	40 fish/L for 3 h	↑ vs. control	[87]
Chronic crowding	40 fish/L for 5 days	↑ vs. control	[87]
Acute stress battery	1 day of net chasing + air exposure + water level decrease + crowding (see ref for further details)	↑ vs. control	[19]
Low- grade CUS	12 days of changes in light intensity and spectrum + pH changes + increased water current + crowding + plastic plant introduction + dissolved food extract (see ref for further details)	Ø vs. control	[19]
High-grade CUS	12 days of changes in lighting schedule + net chasing + net restraint + air exposure + crowding + water level decrease + isolation (see ref for further details)	↑ vs. control	[19]

↑ increased/activated, ↓ reduced/inhibited/impaired, Ø no effect, *LSD* lysergic acid diethylamide, *PCP* phencyclidine, *CDP* chlordiazepoxide, *CUS* chronic unpredictable stress

exposure resulted in a robust anxiety-like behavioral response, notably represented through significantly decreased exploration and increased erratic movements and freezing bouts in the novel tank test (Figs. 4 and 6, Table 3) [24]. In contrast, chronic alarm pheromone produces no changes in fish, suggesting that alarm pheromone is only effective acutely, most likely reflecting its natural use as a fast-acting danger signal to nearby shoals [76]. Another study found that acute hypoxanthine 3-N-oxide, a molecule common to the alarm pheromones secreted by several fish species, elicited more erratic movements and jumps as the dose increased [100].

The presence of a predator is another universal stressor for animals [101, 102]. Zebrafish display significant behavioral response to a variety of predator stimuli, such presence and visualization of a sympatric predator, the Indian leaf Fish (*Nandus nandus*, also known as the Gangetic leaf fish). For instance, visual exposure to the Indian leaf Fish resulted in zebrafish geotaxis, unusual tightened grouping (shoaling) of conspecifics and avoidance of the predator by gathering to the opposite corner (Fig. 6) [99]. Experimentally naïve zebrafish respond significantly stronger to a *sympatric predator* from their natural habitat than to an *allopatric predator* (i.e., compressed cichlid, from different non-overlapping natural habitats), suggesting a genetically based predator anxiety [102]. Both acute and chronic exposure to the Indian leaf fish produced similar behavioral responses in the novel tank test [76]. Notably, although the zebrafish displayed a typical response to stress with an

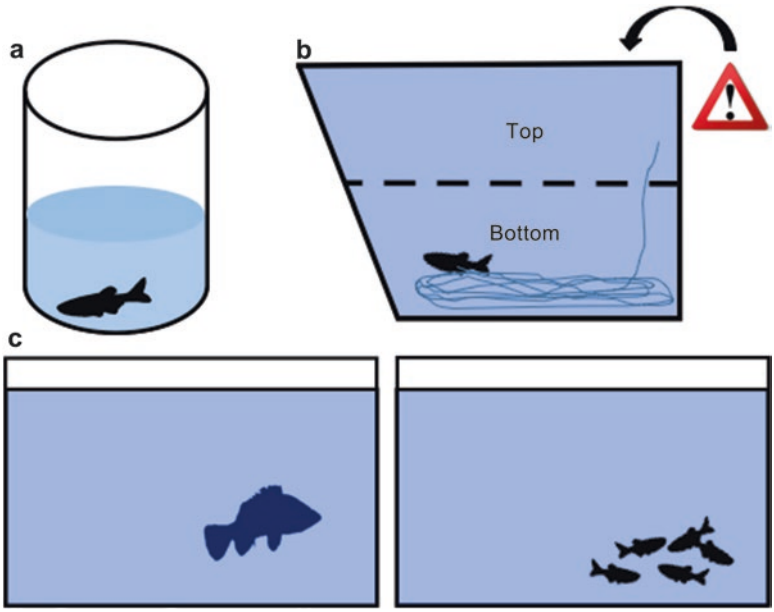

Fig. 6 Experimental stressors commonly used to trigger and assess anxiety-like behaviors in adult zebrafish. (**a**) The beaker stressor test. In this model, zebrafish are removed from their home tank and placed individually in 100 mL of water within a 250 mL beaker for 15-min, resulting in elevated whole-body cortisol levels and anxiety-like behaviors [8]. (**b**) Alarm substance exposure in the novel tank test. Alarm substance is extracted from epidermal cells of euthanized zebrafish and administered to the novel tank test water prior to testing, resulting in increased anxiety-like behaviors [24]. (**c**) Predator exposure paradigm. In this model, a zebrafish tank is placed adjacent to a tank containing a big predator fish (e.g., Indian leaf fish, a natural predator of zebrafish). Visual exposure to a predator fish results in avoidance, tightened shoal cohesion and bottom dwelling [99].

increase of erratic movements, they also displayed shorter latency to enter the upper half and more time spent in the upper half, which are not characteristics associated with stress in the novel tank paradigm. However, as the predator fish spent the majority of the time in the bottom of the tank, it appears that the zebrafish displayed a distinct learned avoidance behavior by moving to the area least likely to be occupied by a predator. In contrast, typical anxiety-like behavior was only significant in the erratic movement endpoint during exposure to an allopatric predator Oscar fish (*Astronotus ocellatus*), indicating weaker responses as compared to Indian leaf fish exposure [76]. This further suggests the importance of a strong genetic 'innate' influence on the zebrafish fear response.

Predator stimuli that are artificial [74], real [8, 99], or computer-generated [103] produce robust and reproducible anxiogenic phenotypes in zebrafish. For example, a recent study found that an animated dot increasing in size presented from above a tank on a computer screen elicited a stronger fear response than other predator-related stimuli (e.g., animated Indian leaf fish, animated needle fish, and a bird

silhouette) [104]. The dot stimulus may mimic an approaching fishing bird, another natural predator of zebrafish [105]. Zebrafish treated with acute anxiolytic dose of ethanol show reduced fear/anxiety behaviors compared to control fish in response to a computer generated moving bird silhouette presented from above the tank, as measured by distance to bottom of tank and erratic movements [104]. The approach of using computer generated predator stimuli is particularly attractive due to the automated delivery and consistency of the stimulus, especially when coupled with automated behavioral quantification software [14, 103, 106].

5 Genetic Manipulations and Strain Differences

Genetic mutations that alter gene expression and disrupt physiological functions of the brain contribute to the pathogenesis of a variety of psychiatric disorders [107]. For example, methyl-CpG-binding protein 2 (mecp2) epigentically regulates human brain development, and the mutations of this gene are attributed to neurodevelopmental disorders, such as Rett syndrome (RTT), X-linked mental retardation and autism spectrum disorder (ASD) [108]. Knockout of *mecp2* in larval zebrafish decreased their locomotor activity levels and average velocity in the open field test compared to wild type larvae [109]. Motor impairment is a phenotype commonly observed in mecp2-related disorders in humans, as well as in rodent models [108]. *Mecp2* zebrafish mutants also showed decreased levels of thigmotaxis in the open field [109], an effect that is inconsistent with rodent *mecp2* mutants [110, 111]. This phenotypic difference may be attributable to the larval motor dysfunction, or an avoidance of tactile stimulation from the wall, similar to hyper-responsive ASD patients [112].

One of the main challenges in zebrafish neurophenotyping research is the relatively limited number of outbred or inbred wildtype 'reference' strains, as compared to nearly a hundred of wild-type strains currently available for mice (www.jax.org). As seen in other species, different genetic strains in zebrafish may contribute to varying behavioral phenotypes. For example, in the novel tank test the wild-caught zebrafish from a small river in Bengal (India) exhibited more anxiety-related endpoints (less top transitions, less time in the top, more freezing bouts and increased latency to enter the top), compared to a short-fin (SF) outbred laboratory strain (Table 4) [45]. The leopard zebrafish strain also showed increased anxiety-like behaviors in the novel tank test compared to the wild type SF strain [24]. However, the leopard strain did not show differences in total distance traveled or average velocity, suggesting that differences in anxiety were not due to motor/neurological deficits. In studies using an animated predator stimulus (i.e., Indian leaf fish) the WIK and TU zebrafish strains showed an atypical preference for the side of the tank where the predator stimulus was presented [113], and the AB strain showed avoidance of the stimulus [114]. In a novel tank test study, the WIK zebrafish spend more time in the top of the tank compared to the AB line, suggesting that the WIK strain may be less anxious compared to others [71], most likely reflecting their genetic closeness to the wild zebrafish. The TM1 strain was more likely to approach an

artificial painted allopatric predator fish model (cichlid, *Etroplus canarensis*) and took less time to recover after being transferred to a new tank, as measured by latency to feed, when compared to the SH and Nadia strains [115]. Clearly, understanding different behavioral profiles between zebrafish strains is an important method to determine the contribution of genetic background on anxiety and stress related behaviors. Combined with the availability of the growing number of transgenic and mutant zebrafish (some of which show overt differences in anxiety-related behaviors and physiology discussed above), the expansion of this effort and the identification of candidate genes or gene loci will aid in determining genetic susceptibility to stressors in humans.

Additional useful approaches to studying zebrafish anxiety-like traits include quantitative trait loci (QTL)-based analyses and the genetic knockdown of various genes. For example, QTL analysis involves crossing two populations or strains and genotyping the intercross generation, which ultimately reveals the relation between a genomic region and a phenotype [116]. QTL analysis of over 100 mouse behavioral phenotypes in the open field test and light dark test detected 17 QTL accounting for phenotypic variation [117]. In zebrafish, an F2 generation derived from crossing a wild Indian strain with the AB strain revealed QTL mapping of anti-predatory behavior (shoaling) and 'boldness' (approach to a novel object) [118]. The genomic region for anti-predatory behavior was located on chromosome 21, and the region for 'boldness' was located on chromosome 9 and 16 [118]. Zebrafish offer great potential for evaluating behavioral phenotypes at the genetic level using QTL mapping due to low-cost and high fecundity, although few QTL studies with zebrafish have been conducted at this point [116, 118, 119]. Gene knockdown technologies in zebrafish are also valuable systems to elucidate vertebrate gene function that can be achieved using a variety of methods such as zinc-finger nucleases [120], transcription activator-like-effector nuclease (TALEN) [27, 121] and clustered regularly-interspaced short palindromic repeats (CRISPR) [121]. For example, CRISPR knockdown of vesicular monoamine transporter 2 (Vmat2) in zebrafish results in decreased levels of dopamine, serotonin, norepinephrine and their metabolites [46]. A similar reduction in monoamines is seen in Vmat2 heterozygous mice [122]. *Vmat2* mutant zebrafish also increased geotaxis in the novel tank test, and female mutants are more anxious than males. Interestingly, chronic pharmacological blockage of Vmat2 in zebrafish by reserpine treatment cased general hypoactivity in zebrafish [123] and elevated cortisol levels, generally consisted with increased affective (albeit not purely or necessarily anxiogenic) tone.

6 Conclusion

Despite anxiety-related disorders being one of the most widespread neuropsychiatric conditions, their pathological mechanisms are poorly understood, and their treatments remain essentially the same for the last 50 years [124, 125]. Discovering the underlying mechanisms of psychopathology is fundamental to treatment, reversal,

or prevention of complex brain disorders, including stress/anxiety-related illnesses [126, 127]. A substantial challenge faced by phenotype-based screening is expensive and inefficient mammalian models that require large quantities of compounds and time during experimentation [124, 125]. With a clear benefit of genetic and physiological similarity, the use of zebrafish as an alternative model mitigates these limitations [128–130]. Recent circuitry-based studies in zebrafish continue to unravel complex neural regulation of anxiety-related states in this species [131, 132]. Together with a robust sensitivity to drugs and acute/chronic stressors, novelty-based paradigms, endocrine correlates, and an ease of genetic manipulation makes high-throughput phenotyping and pharmacological screens in zebrafish a promising possibility in translational neuroscience of anxiety "from tank to bedside" [8, 133, 134].

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